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Multiple Shoot Regeneration of Roselle (*Hibiscus sabdariffa* L.) from a Shoot Apex Culture System

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Abstract: The present study reports a protocol for the efficient *in vitro* regeneration of *Hibiscus sabdariffa* using the shoot apex as explant. Shoot tips were cut 10 days after germination on MS medium complemented with 4.43 µM of 6-benzyladenine (BA). Different concentrations of BA (8.87-35.48 µM), thidiazuron (0.91-18.16 µM) and *m*-topolin (8.28-33.12 µM) in the MS medium were assessed for shoot production. A considerable variability in regeneration efficiency among the growth regulators used was observed as shoots developed over the apical meristem in a rosette disposition. The highest number of shoots (33) was obtained with the 17.74 µM BA treatment, compared to the 16 shoots obtained with 16.56 µM *m*-topolin. The shoots obtained were placed in MS media without growth regulators in total darkness to induce stem elongation. Production of roots was abundant, obtaining 98.6% of regenerated plants with the BA and *m*-topolin treatments. The genetic variation analysis through RAPD showed a uniform polymorphic pattern to the reganerated plants indicating that they were genetically homogeneous. This research is the first report of the inductive effect that BA has on the production of multiple plants from a shoot apex of *H. sabdariffa* and the possible regeneration of plants without a rooting medium.

Key words: Cytokinins, regeneration, multiple buds, RAPD, *ex vitro*

INTRODUCTION

Roselle (*Hibiscus sabdariffa* L.) is a dicot plant of the Malvaceae family originating from Africa. It is currently grown in tropical regions of India and parts of Asia, Australia and America. The calyx is widely used for producing drinks or tea due to its high content of anthocyanins, particularly cyaniding-3-sambubioside, delphinidin-3-sambubioside and delphinidin-3-glucose (Hong and Wrostlad, 1990) and organic acids such as ascorbic, malic, tartaric and hibiscus acids, which give roselle drinks their palatable sour taste. It is also used as flavoring and color additive in the manufacture of jam, liquor and jellies (Akindahunsi and Olaleye, 2003). In several countries it is used as a natural medicine for treating hypertension (Faraji and Tarkhani, 1999), pyrexia and liver disorders (Chen *et al.*, 2003) and microorganism growth limitation (Oboh and Elusiyan, 2004), as well as a diuretic, digestive and sedative (Akindahunsi and Olaleye, 2003). Pharmacological studies of anthocyanins in hibiscus have shown that they have antioxidant activity in patients with atherosclerosis (Tsai *et al.*, 2002). The plant grows as an annual and sometimes biannual shrub with straight branches and small ramifications, with yields that can reach 0.5 to 2 t ha^{-1} depending on the variety. Cultivated plants reach between 1 and 3 m in height depending on the location and season of sowing. The crop is susceptible to the attack of various plant pathogens such as *Phytophthora parasitica*, *Phoma sabdariffae*, *Macrophomina phaesolina*, *Rhizoctonia solani*, *Botrytis cinerea*, *Sclerotium rolfsii* and *Coniella musaiaensis* var. *hibisci* (Persad and Fortune, 1989; Swart and Langenhoven, 2000), which can infect plants at early development stages, when competition from weeds can also be deleterious.

There are few reports on the *in vitro* regeneration of the *Hibiscus* genus, with kenaf (*H. cannabinus* L.) being the most studied species in terms of

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organogenesis regeneration from apex (Zapata *et al.*, 1999; Srivatanakul *et al.*, 2000), while other authors report the formation of multiple buds from young buds and cotyledons (Khatun *et al.*, 2003; Herath *et al.*, 2004). A protocol for plant regeneration from calli using segments of hypocotyls and cotyledons has been established for the *Hibiscus siryacus* species (Jenderek and Olney, 2001). Currently there are no reports describing methods for the *in vitro* regeneration of *Hibiscus sabdariffa*. Hence, development of suitable regeneration methods would be important for the improvement of the existent cultivars of roselle. The purpose of this study was to develop a reproducible system for regenerating *H. sabdariffa* plants through multiple buds growing from a shoot apex, using BA, *m*-topolin and thidiazuron (TDZ).

MATERIALS AND METHODS

Seeds germination and explant generation: This study was carried out in the Laboratory of Molecular Biology of the Technological Institute of Tlajomulco, Jalisco, Mexico. Seeds of the roselle variety Colima were provided by the INIFAP in Tecoman, Colima, Mexico. Seeds were surface sterilized by immersion in 70% ethanol for 3 min followed by 20 min in a 50% (v/v) sodium hypochlorite solution plus 0.2% Tween 20 with constant stirring and finally rinsed 3 times with sterile distilled water. Sterilized seeds were placed on MS medium (Murashige and Skoog, 1962) plus 4.43 μM of BA and incubated at 27°C with continuous light. Shoot apex (5-6 mm) with two leaf primordia and nodal segments which were excised from 10-days-old seedlings grown *in vitro* were used as explants source.

Multiple shoot induction: The shoot tips were placed vertically on Sprouting Induction Medium (SIM) containing Murashige and Skoog (1962) salts supplemented with 555 μM of *myo*-inositol, 1.5 μM thiamine HCl, 4.1 μM nicotinic acid, 2,4 μM pyridoxine HCl, 3% sucrose and plant growth regulators. The pH of the medium was adjusted to 5.7 with NaOH and 0.35% of phytagel was added as a jellifying agent. Various concentrations of BA (8.87, 17.74 and 35.48 μM), *m*-topolin (8.28, 16.56 and 33.12 mM) and thidiazuron (0.91, 2.27, 4.54, 9.08 and 18.16 μM) were used for multiple shoot induction. After the jellifying agent was dissolved, 20 mL of medium was placed in jars and sterilized in autoclave at 121°C for 20 min. Treatments were maintained at 28±2°C and a 16/8 h photoperiod with a light intensity of 25 μmol m^{-2} sec^{-1}.

A total of 9 repetitions were used for each treatment and 10 apical buds were placed in each culture jar. Once

bud formation started, they were sub-cultured every 2 weeks for a total time of 12 weeks. A control group was kept in MS medium without growth regulators to standardize the response. Experiments were established in a full random design registering the frequency and number of buds formed by each explant. An ANOVA was used to assess variance between treatments and Duncan's Multiple Range Test was used to assess the distance between means at 5% significance.

Bud elongation, rooting and transfer of the plantlets to soil: Each group of differentiated buds was elongated in total darkness for 3 to 4 days and once they reached 3 to 4 cm in length they were cut into individual sections and placed in MS media without growth regulators to promote vegetative and radicular development. Regenerated plants were transferred into growth jars with a sterile mixture of perlite and Sunshine® (1:1 v/v) and 50% liquid MS without sucrose. A cellophane cover was placed to promote the gaseous exchange; after three weeks in laboratory conditions, the cover was removed and the plantlets were transferred to pots containing sterile soil and subsequent grown in a green house. After 4 weeks, they were transferred to the field, where they completed their vegetative cycle and calyx production.

DNA extraction and RAPD analysis: Genomic DNA was extracted from approximately 100 mg of leaf tissue using the CTAB method (Doyle and Doyle, 1990). RAPD analyses were conducted on each plant produced from the BA and *m*-topolin treatments, in order of detect any somaclonal variation. The reliability of 20 arbitrary 10 base primers for RAPD analyses was initially tested using Kit B (Operon Technologies). At least two independent PCR runs were performed per sample and selected primer. Only fragments amplified with similar staining intensity in each of these runs were considered in subsequent analyses. For each primer, RAPD bands were scored as present or absent by visual inspection of gel photographs and the molecular weight of each fragment was estimated from digitalized gel images. PCR amplifications were carried out in a Techne Genius Thermocycler using a 25 μL reaction mixture composed of Tris-HCl 20 mM pH 8.4, KCl 50 mM, MgCl$_2$, 2 mM, dNTPs 0.2 mM each one (Invitrogen), decanucleotide primer 0.5 μM (Operon Technologies), 50 ng of genomic DNA and 0.5 U *Taq* DNA polymerase (Invitrogen). Reaction conditions consisted of an initial denaturing at 94°C for 1 min, followed by 35 cycles of 60 sec at 94°C, 60 sec at 36°C and 90 sec at 72°C and a final extension step of 7 min at 72°C. Amplicons were separated on 1.2% agarose gel in SB buffer (Sodium

Borate 10 mM, pH 8.0) and visualized by ethidium bromide staining under UV light, their sizes were estimated using a 100 bp ladder (Invitrogen).

RESULTS AND DISCUSSION

Preliminary studies assessed the effect of α-Naphtalene Acetic Acid (NAA) (10.74-42.96 μM) and BA (8.87-35.48 μM) in the induction of somatic embryogenesis in discs of cotyledon leaves and hypocotyls. This experiment was unsuccessful since callus formation with abundant root production was the only effect observed when NAA and BA concentrations higher than 20 μM and lower than 10 μM were used, respectively. The best growth regulators combination for producing friable callus was obtained after 3 weeks by combining hypocotyls as explants with 21.48 μM of NAA and 35.48 μM of BA (data not shown). McLean *et al.* (1992) reported that a concentration of 0.54 μM NAA and 0.44 μM BA induces the production of adventitious buds in calli of *H. cannabinus* after 4 weeks, these conditions could be evaluated in latter studies of roselle.

Sprouting of seeds in medium with 4.43 μM BA facilitates isolation of the explant due to the shortening and widening of the apical bud, as well as the absence of root production when compared to untreated plants. This treatment also predisposes the explant to earlier bud formation.

The cytokinins used, in general, showed different effects in apical bud formation (Table 1). In treatments with BA, the sprouting process started 3 weeks after transplant into SIM medium and increased notably with the formation of a large number of multiple buds in the medium supplemented with 17.74 μM BA, in which an average of 33.2 buds per explant were obtained after 8 weeks (Fig. 1a), compared to 2.7 and 3.2 buds with 8.87 and 35.48 μM BA, respectively (Table 1). With the use of *m*-topolin, the appearance of buds started one week earlier than with BA, although after 8 weeks on medium supplemented with 16.56 μM *m*-topolin a lower average number (16.1) of buds per explant were obtained. In general, new buds formed when BA and *m*-topolin were combined, forming a rosette and regenerating directly without the formation of a callus (Fig. 1b). Treatments with TDZ did not show bud formation and explants developed a vitrified callus and foliar deformation at the apical bud. These results differed from those reported for kenaf, where the addition of 1 μM of TDZ induced the production of 6 buds in 2 weeks, using apical buds as explants (Srivatanakul *et al.*, 2000). In light of these

Table 1: Effect of different cytokinins on multiple shoots induction from isolated shoot apex explants of *H. sabdariffa* 12 weeks after induction

Treatments	Cytokinins (μM)	No. of shoots per explant[a]	Shoot regeneration efficiency (%)[b]
1	Control	No regeneration	No regeneration
BA			
2	8.87	2.7d	99a
3	17.74	33.2a	100a
4	35.48	3.2d	99a
***m*-topolin**			
5	8.28	4.5c	93b
6	16.56	16.1b	99a
7	33.12	3.7dc	97a
Thidiazuron			
8	0.91	No regeneration	No regeneration
9	2.27	0.08e	0c
10	4.54	0.01e	0c
11	9.08	No regeneration	No regeneration
12	18.16	0.05e	0c

[a]Means from 90 trials. Means within a column followed by different letter(s) are significantly different from each other at the 5% level with Duncan's Multiple Range Test, [b]Plants regenerated/bud explants X 100

results we do not recommend the use of TDZ for micropropagation of roselle in the concentrations herewith employed. Treatments with BA and *m*-topolin showed a greater than 98% frequency of sprouting using apical buds as explants. Other roselle explants, such as leaves or hypocotyls, form a callus grown under the same cytokinin concentrations.

Buds were cut once when they had approximately 1 to 4 cm in length, which was obtained by keeping the rosette in darkness for 4 days, thus inducing stem elongation. Buds that were cut with approximately 6 leaves showed a well developed radicular system (Fig. 1c) making it unnecessary to provide a root formation medium. This condition is possibly due to a high content of endogenous auxins. After the explants had been established for 2 weeks in hormone free MS medium, the plants were vigorous and had an abundant radicular system (Fig. 1d) which facilitated their adaptation to the soil substrate, where they continued their growth without any apparent acclimatizing problems (Fig. 1e).

One-hundred percent of the cut buds managed to develop fully in field conditions with phenotypic conditions similar to roselle plants of the same variety that originated from seeds, in which calyx formation was observed at 3 months after their establishment (Fig. 1f). Buds cut from explants in *m*-topolin media had a decreased root formation when compared to those obtained from the BA medium; nevertheless, they did not show any problems for *ex vitro* adaptation.

To our knowledge there are no reported references on the regeneration of *H. sabdariffa*. Nevertheless, BA and *m*-topolin auxins produce a good bud sprouting response

Fig. 1: Stages of multiple shoots formation from roselle (*Hibiscus sabdariffa* L.). Bar = 1 cm. (a) proliferative stage, 3 weeks after sprouting in induction media with 17.74 µM BA; (b) shoots, 4 weeks after being established in SIM, following a period of darkness to induce stem elongation; (c) elongated shoots, cut and placed in MS medium; (d) development of vigorous roots and elongation of the stem in MS medium; (e) substrate adaptation phase; (f) establishment and production of calyces in the field

compared to other Malvaceae such as *H. cannabinus*, in which the application of 8.8 µM of BA produced 11 buds, while higher concentrations, up to 17.6 µM, significantly reduced this number to 4 per explant (Herath *et al.*, 2004). Furthermore, the combination of 22.19 mM BA and 2.85 mM NAA plus 0.5% Puronic F-68, a surfactant, produced up to 11 buds per explant (Khatun *et al.*, 2003), while concentrations of 0.44 µM of BA did not produce any bud formation response (Zapata *et al.*, 1999).

Development of axillary meristems is controlled by the inhibiting effect exerted by the apical meristem; nevertheless, it is possible that exposure to BA or *m*-topolin reprograms the cells of axillary meristems in young buds, causing accelerated division giving rise to numerous vegetative buds. A gene was recently discovered in *Arabidopsis thaliana*, known as *BRANCHED1* (BRC1), that is similar to the *Teosinte branched1* (*tb*1) found in corn, which codifies for a TCP family-type transcription factor. This gene is required for inducing auxin-type apical dominance; therefore, it regulates negatively the formation of axillary buds (Aguilar-Martínez *et al.*, 2007). This suggests the possibility that a homologous gene might exist in *H. sabdariffa* that could be similarly suppressed by the action of BA or *m*-topolin cytokinins, leading to the induced production of multiple buds in shoot tips. Recent studies using light and scanning electron microscopy in *H. cannabinus* have shown that regeneration in multiple buds originating from an apical bud treated with BA starts with the formation of meristem regions adjacent to axillary buds and on the abaxial surface of the primary leaf primordia which later develop into shoots (Herath *et al.*, 2004).

Regarding the genetic variance assayed through RAPD analyses, a total of 77 fragments were amplified ranging from 1.6 to 0.3 Kbp and averaging 5 bands per primer set. The best primers were OPB5 and OPB11, which showed a monomorphic pattern in plants that were regenerated with BA or *m*-topolin when compared to the group not treated with growth regulators (data not shown). Based on these results, we suggest that there is no somaclonal variation in the regenerated plants, possibly due to the fact that they were directly regenerated from an organized bud tip.

With these results we can state that it is possible to carry out genetic transformation of *H. sabdariffa* through particle bombardment or through *Agrobacterium*, using young shoots as target. After transformation, additional treatment with BA could contribute to bud proliferation and, in consequence, prevent the appearance of chimerical shoots, thereby leading to more stable transformations.

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